Vitamin D: A Hormonal Regulator of the cAMP Signaling Pathway

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CONTENTS

I. INTRODUCTION

3′,5′-cyclic Adenosine monophosphate (cAMP), a universal intracellular second messenger molecule, is employed by several neurotransmitters, biogenic amines, peptides, and glycoprotein hormones. Ligand-receptor coupling in the cell membrane activates a guanine nucleotide binding protein (G-protein) which stimulates the formation of cAMP from adenosine triphosphate by the adenylyl cyclase (AC).[1] Cyclic AMP activates the cAMP-dependent protein kinase (PKA) and initiates a cascade of protein phosphorylations which alter the expression of cAMP-regulated genes and subsequently cell proliferation and differentiation.[2,3] Vitamin D is another regulator of cell growth and function in several cells.[4] In this chapter we report that vitamin D regulates the thyroid-stimulating hormone (TSH)/cAMP signaling pathway in a rat thyroid cell line (FRTL-5).

II. THE VITAMIN D ENDOCRINE SYSTEM

Vitamin D_3 (cholecalciferol), formed in the skin from 7-dehydrocholesterol or provided by the diet, is 25-hydroxylated in the liver. The product formed, calcidiol (25-hydroxycholecalciferol), is subsequently 1α-hydroxylated in the kidneys to the biologically most active form of vitamin D_3, calcitriol (1α,25-dihydroxycholecalciferol). Alternatively, calcidiol is 24-hydroxylated to 24*R*-hydroxycalcidiol (24,25-dihydroxycholecalciferol), which does not bind to the vitamin D receptor (VDR) at physiological concentrations. The formation of 24*R*-hydroxycalcidiol is facilitated by high serum levels of calcitriol and Ca^{2+}, that at the same time attenuates the activity of the 1α-hydroxylase constituting a classical negative-feedback loop.

The VDR is an intracellular protein consisting of a carboxy-terminal segment binding calcitriol and a smaller amino-terminal DNA-binding domain.[5] The cloning and sequencing of the VDR demonstrated that it belongs to the superfamily of thyroid and steroid hormone receptors.[6,7]

Ligand-activated VDR binds to double-stranded chromatin and regulates gene transcription through *cis*-acting vitamin D responsive elements (VDREs) in the 5′-flanking region of the gene.[5,8] The arrangement of the VDRE motif determines whether the VDR binds to the VDRE as a homodimer or as a heterodimer with a receptor auxiliary factor.[5,9–11]

The main function of the vitamin D endocrine system is to regulate Ca^{2+} homeostasis by controlling cellular processes in intestine, kidneys, and bone.[4] VDRs were therefore initially demonstrated in tissues involved in the regulation of mineral homeostasis. However, VDRs and effects of vitamin D have been

demonstrated in several other cells and tissues not primarily involved in the regulation of Ca^{2+} metabolism.

Calcitriol induces differentiation of hematopoietic cells to monocytes/macrophages, fused macrophages, granulocytes, and osteoclasts.[4,12] In cultured rat pituitary cells (GH_3), calcitriol attenuated prolactin production at physiological Ca^{2+} concentrations, whereas prolactin production was stimulated at low Ca^{2+} concentrations.[13] Calcitriol has been shown to attenuate the growth of breast and colon carcinomas, lymphomas, and leukemias, but its therapeutic use has been hampered by the induction of hypercalcemia.[14]

In an attempt to reduce the hypercalcemia and to augment the effect on growth inhibition and differentiation several vitamin D analogs have been synthesized.[15] Since only one VDR is known, the strategy has been to synthesize analogs with increased metabolic clearance rate. Vitamin D is bound to the vitamin D-binding protein (DBP) in serum, and by altering the side chain of the analog the binding affinity to DBP is reduced.[16] One such analog is calcipotriol (MC 903) which is used in the treatment of psoriasis.[17] Vitamin D analogs have also been tried in the treatment of breast carcinomas and as immunosuppressive agents.[14]

III. EFFECTS OF TSH/cAMP IN THYROID CELLS

Thyrotropin (TSH) is the major regulator of thyroid cell growth and differentiation.[18] TSH binds to and activates a TSH receptor (TSHR) located in the cell membrane. The activated receptor activates the α subunit ($G_s\alpha$) of the AC stimulatory G-protein inside the cell. The AC is stimulated by $G_s\alpha$ and forms cAMP from adenosine triphosphate. Most effects of TSH on thyroid cells may be mimicked by cell permeable cAMP analogs. Cyclic AMP stimulates both cell growth and cellular functions ultimately leading to the synthesis of thyroid hormones.

A. THE FRTL-5 CELL LINE

The rat thyroid cell line FRTL-5 is a strain of continuously growing cells which have retained a functional TSHR (Figure 1).[19,20] TSH stimulates the intracellular cAMP production, cell growth, iodide uptake, and thyroglobulin synthesis, but they have lost the ability to produce thyroid hormones.[20]

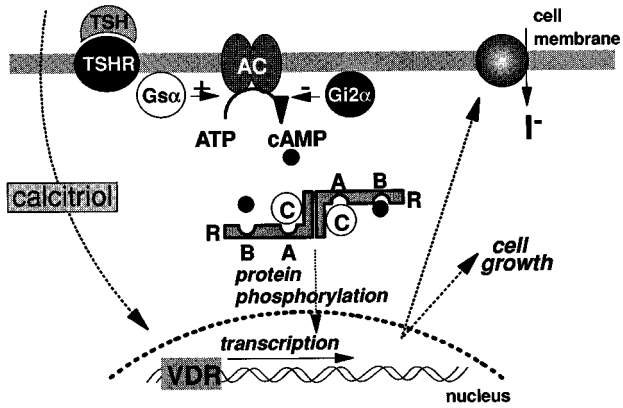

Figure 1 TSH and calcitriol stimulation of FRTL-5 cells. TSH binds to and activates TSH receptors (TSHRs) in the cell membrane. The activated TSHR activates the adenylyl cyclase (AC) stimulatory G-protein (Gsα), and cAMP is formed from adenosine triphosphate (ATP). Cyclic AMP binds to sites A and B of the regulatory subunits (R) of the PKA and liberates the catalytic subunits (C), which induces protein phosphorylations and stimulates I^- uptake and cell growth in processes requiring protein synthesis. The AC is inhibited by inhibitory G-proteins (Gi2α). Calcitriol readily passes the cell membrane and regulates gene transcription by binding to an intracellular vitamin D receptor (VDR).

1. Vitamin D Attenuates TSH-Stimulated cAMP Production

FRTL-5 cells express an intracellular specific VDR, and when FRTL-5 cells were treated with calcitriol, both basal and TSH-stimulated cAMP production were attenuated.[21–23] Maximum effect was obtained after 3–4 days of incubation with 10 n*M* calcitriol, and recovery of the cAMP response after cessation

of calcitriol treatment required about 8 days. This slow response is typical for hormones that regulate cell function by stimulating or inhibiting gene expression. The effects of calcidiol and 24R-hydroxycalcidiol on TSH-stimulated cAMP production were minimal or absent and correlated to their low binding affinities for the VDR.[21,23]

2. Effects of Vitamin D Analogs

Calcitriol inhibited the TSH-stimulated cAMP production and subsequently the TSH-stimulated iodide uptake and cell growth. Similar effects were observed when the side chain modified vitamin D analogs MC 903, KH 1060, and EB 1089 were used.[24] KH 1060 was the most potent inhibitor, in agreement with the observation that the concentration of KH 1060 needed to induce 50% displacement of [^{3}H]calcitriol binding to the VDR was lower for KH 1060 than the other analogs. In general, the biological effects of the analogs correlated to their VDR binding affinity. However, MC 903 was the second most potent inhibitor of cell growth in spite of having the lowest affinity for the VDR. Nongenomic effects of vitamin D have been demonstrated in several cells.[25] It is possible that the increased growth inhibitory effect of MC 903 in FRTL-5 cells is partially mediated by nongenomic mechanisms.

3. Calcitriol Regulates the Transmembrane Signaling of TSH

To attenuate the TSH-stimulated cAMP production calcitriol may inhibit the transduction of the TSH signal at the levels of the TSHR, the AC stimulatory G-proteins, or the AC. In addition, AC inhibitory G-proteins and G-protein βγ subunits may modulate the AC activity.[26]

The TSHR is an important marker of a differentiated thyroid cell.[18] When FRTL-5 cells were cultivated in the absence of TSH, the TSH-stimulated AC activity increased, and was associated with an increased TSHR level.[27] Calcitriol treatment of the cells attenuated this increase in AC activity (Figure 2).[28] Scatchard analysis showed that the receptor number was 56% lower in the calcitriol-treated cells when compared to the control cells. TSH removal caused a sudden drop in the TSHR mRNA levels in both control and calcitriol-treated cells. This was probably related to reduced intracellular cAMP levels. It has been shown that the 5′-flanking region of the rat TSHR gene contains a cAMP responsive element.[29] Twelve hours after the TSH removal the TSHR mRNA level increased in the control cells, whereas it continued to be suppressed in the calcitriol-treated cells. TSH may induce the synthesis of transcription factor(s) that attenuates the expression of the TSHR.[30]

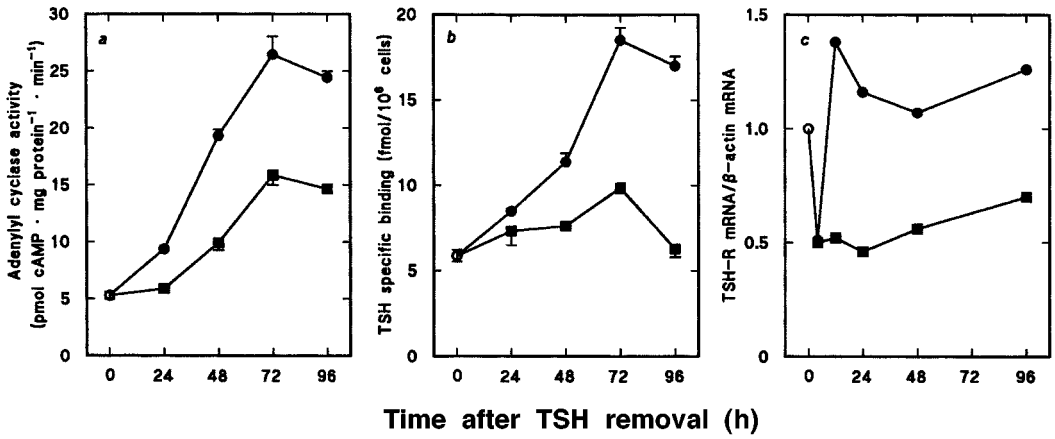

Figure 2 TSH stimulated adenylyl cyclase activity, TSH receptor binding, and mRNA levels in FRTL-5 cells after removal of TSH from the culture medium. The cells were pretreated in culture medium containing 1 U/l TSH. At time zero the cells were incubated in culture medium without TSH and 10 n*M* calcitriol (■) or vehicle (●) were added. At the time indicated adenylyl cyclase activity stimulated with 50 U/l TSH (a), specific binding of TSH (b), and TSH receptor (TSH-R) mRNA contents correlated to β-actin mRNA levels (c) were measured.[28]

Calcitriol attenuated both basal as well as forskolin and to some extent cholera toxin stimulated AC activity in FRTL-5 cells.[28] This indicated inhibitory effects of calcitriol distal to the TSHR. Protein or mRNA levels of the AC stimulatory G-protein $G_s\alpha$ were not affected by the calcitriol treatment. Three

AC inhibitory G-proteins ($G_{i-1}\alpha$, $G_{i-2}\alpha$, and $G_{i-3}\alpha$) have been characterized, and they are encoded by different genes.[1,31] Calcitriol induced a 4-fold increase in $G_{i-2}\alpha$ at both the mRNA and protein levels in FRTL-5 cells, whereas $G_{i-3}\alpha$ was unaffected (Figure 3).[28] FRTL-5 cells have both purinergic P_1 and somatostatin receptors, which may stimulate $G_{i-2}\alpha$.[32,33] $G_{i-1}\alpha$ is not expressed in FRTL-5 cells.

Regulation of $G_{i-2}\alpha$ has been associated with altered AC activity, cell differentiation, and proliferation. Mutations rendering $G_{i-2}\alpha$ constitutively active (*gip2*) were shown to be oncogenic in some endocrine tumors.[34] Increased $G_{i-2}\alpha$ expression or *gip2* gene transfections have been shown to increase the proliferation rate of Rat-1 fibroblasts, NIH 3T3 fibroblasts, and the metastatic potential of K1735 murine melanoma cells.[35–37] Blocking the translation of $G_{i-2}\alpha$ in liver and adipose tissues in transgenic mice by the expression of $G_{i-2}\alpha$ antisense oligonucleotides reduced both liver and fat mass expressed as a percentage of body weight.[38]

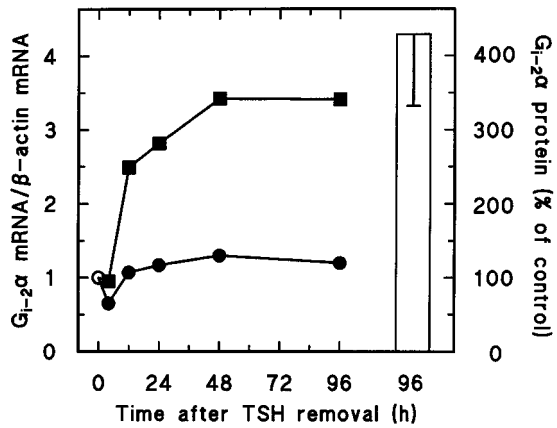

Figure 3 $G_{i-2}\alpha$ mRNA and protein levels in FRTL-5 cells. The cells were pretreated in culture medium containing 1 U/l TSH. At time zero the cells were incubated in culture medium without TSH and 10 n*M* calcitriol (■) or vehicle (●) were added. At the time indicated $G_{i-2}\alpha$ mRNA contents correlated to β-actin mRNA levels were measured. G_{i-2} protein was measured in calcitriol-treated cells after 96 h of incubation and correlated to corresponding control cells as indicated by the bar.[28]

Depending on the cell type, cAMP may inhibit or stimulate cell proliferation. In FRTL-5 cells the TSH-stimulated cell growth is attenuated by calcitriol since the effect of TSH, which is the main growth stimulus, is inhibited.[23] However, serum-stimulated FRTL-5 cell deoxyribonucleic acid (DNA) synthesis was augmented by calcitriol.[39] It is generally thought that G-proteins are activated by members of the large family of seven-transmembrane spanning receptors.[1] Insulin-like growth factor-II (IGF-II) receptors have only one single transmembrane domain, but were shown to activate $G_{i-2}\alpha$ by a 14-amino acid residue of the intracellular part of the receptor.[40] In FRTL-5 cells insulin-like growth factor-I (IGF-I) receptors may activate G_i-proteins.[41] Adenosine, which activates G_i-proteins, inhibited the stimulatory effect of TSH on DNA synthesis in FRTL-5 cells, whereas the effect of IGF-I was augmented.[42] The coupling between IGF receptors and $G_{i-2}\alpha$ activation indicates that the upregulation caused by calcitriol may potentiate the effect of IGF-I in FRTL-5 cells.

G-protein βγ subunits also regulate AC activity depending on the AC subtype.[26] AC types 1, 5, and 6 are inhibited by βγ subunits, whereas type 2 is stimulated. Calcitriol did not regulate the β subunits at the protein level in FRTL-5 cells.[28]

Forskolin stimulates the AC directly, and forskolin-stimulated AC activity was attenuated by calcitriol, whereas the effect of cholera toxin, which stimulates $G_s\alpha$, was only modestly inhibited.[28] The inhibitory effect of $G_{i-1}\alpha$ on AC activity has been shown to depend on the AC subtype. AC type 1 activity stimulated by cholera toxin is less potently inhibited by $G_{i-1}\alpha$ when compared to the inhibitory effect on forskolin stimulated activity.[43] This indicates that FRTL-5 cells contain AC type 1.

4. Calcitriol Alters the Effect of cAMP

By attenuating the TSH-stimulated cAMP production in FRTL-5 cells, calcitriol subsequently reduced the TSH-stimulated cell growth and iodide uptake.[23] However, when the cells were stimulated with a

cAMP analog which is cell membrane permeable and hydrolysis resistant, calcitriol still attenuated both cell growth and iodide uptake.[44,45] Apparently there were effects of calcitriol distal to the formation of cAMP.

Cyclic AMP primarily activates PKA, which is a tetramer and consists of two regulatory (R) and two catalytic (C) subunits.[3] Type I PKA (PKAI) contains R subunit type I dimers and type II (PKAII) contains R subunit type II dimers.[46] Alpha and β subtypes of both RI (RIα and RIβ) and RII (RIIα and RIIβ) have been identified. Three C subunits have been characterized (Cα, Cβ, and Cγ).

Two cAMP binding sites, designated site A and B, are found in the carboxy-terminal half of each R subunit.[47] Only site B is exposed for cAMP binding on the inactive PKA tetramer. Binding of cAMP to site B facilitates the binding of cAMP to site A in a positive cooperative fashion. The binding of four cAMP molecules to a PKA tetramer liberates the two C subunits which become catalytically active phosphotransferases and phosphorylate serine or threonine residues on target molecules.

When FRTL-5 cells were treated in the presence of the cAMP analog 8-(6-chlorophenylthio)cAMP (8-CPTcAMP), calcitriol upregulated the PKA subunit RIIβ at both the mRNA and protein levels without altering the levels of the other PKA subunits (Figure 4).[48] Since C subunits preferentially associate with RII subunits, calcitriol increased the level of $RII\beta_2C_2$ tetramers and reduced the formation of $RI\alpha_2C_2$ tetramers.[49] The RIβ subunit is not expressed in FRTL-5 cells.

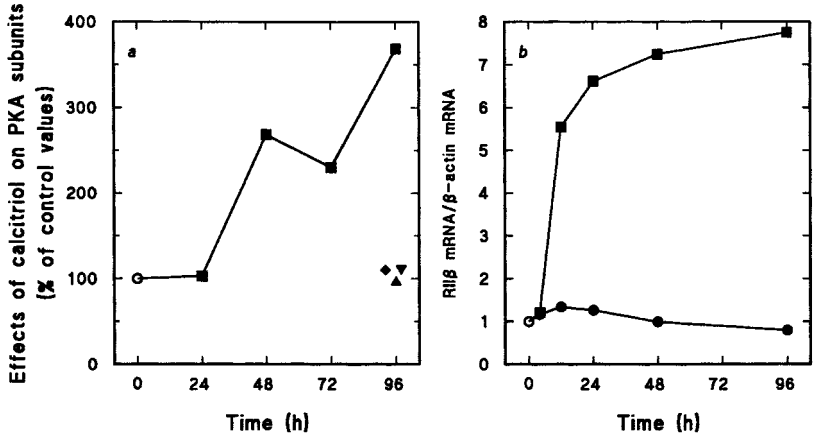

Figure 4 Regulation of RIIβ subunit mRNA and protein levels by calcitriol in FRTL-5 cells. The cells were incubated in culture medium containing 100 μ*M* 8-(6-chlorophenylthio)cAMP in the absence (●) or presence (■) of 10 n*M* calcitriol. At the time indicated RIIβ (■), RIα (▼), RIIα (▲), and C (♦) subunit protein (a) or RIIβ mRNA (b) levels were measured. The protein levels are expressed as a percentage of the corresponding controls.[48]

Effects of calcitriol on cell function were studied by stimulating the cells with PKA type I or type II selective pairs of cAMP analogs. Binding affinities of various cAMP analogs to sites A and B of RI and RII subunits differ. By combining pairs of cAMP analogs PKAI or PKAII can be synergistically activated (Figure 5).[50,51] Calcitriol attenuated the PKAI stimulated iodide uptake in FRTL-5 cells, whereas the effect of PKAII stimulation was unaltered (Figure 5).[48] This indicates that the effect of PKAI stimulation is reduced by increasing the level of RIIβ subunits.

In RIIβ overexpressing NIH 3T3 cells, PKAII levels were increased and the PKAI tetramer eliminated.[52] Cyclic AMP analogs selective for PKA type I or type II activation both stimulated the transcription of a cAMP responsive reporter gene in the control cells. Type I synergism was lost when the cells overexpressed RIIβ subunits. RIIβ subunit expression is cell specific, and regulation of RIIβ has been associated with differentiation of ovarian cells and adipocytes.[3]

IV. VITAMIN D AND cAMP SIGNALING IN NON-THYROID CELLS

The first report of an interaction between calcitriol and the cAMP signaling system was presented by Wong et al.,[53] who showed that calcitriol suppressed the parathyroid hormone-stimulated cAMP

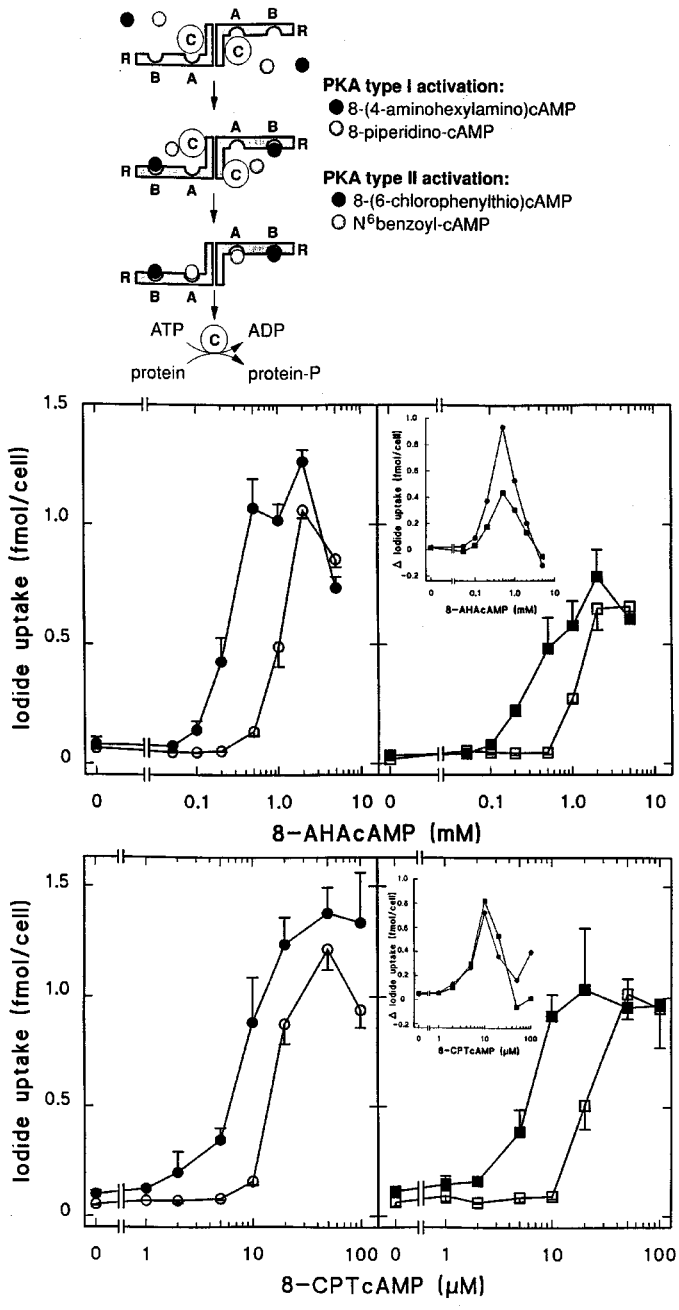

Figure 5 PKA subunit specific stimulation of iodide uptake in FRTL-5 cells. The cells were preincubated for 4 days in culture medium without TSH to reduce the iodide uptake to a minimum and added site B and site A binding cAMP analogs as indicated in the upper panel to stimulate PKA type I or type II. Iodide uptake was measured after 4 days of incubation in the presence (squares) or absence (circles) of 10 n*M* calcitriol. PKAI was stimulated by treating the cells with increasing concentrations of 8-(4-aminohexylamino)cAMP (8-AHAcAMP) in the absence (open symbols) or presence (filled symbols) of a minimally stimulatory concentration of 8-piperidino-cAMP (100 μ*M*) (*middle panel*), while PKAII was stimulated with 8-(6-chlorophenylthio)cAMP (8-CPTcAMP) in the absence (open symbols) or presence (filled symbols) of a low concentration of N^6benzoyl-cAMP (lower panel). The increase in iodide uptake induced by the PKA site A binding cAMP analogs (8-piperidino-cAMP and N^6benzoyl-cAMP) in control (●) and calcitriol (■) treated cells is presented in the insets.[48]

production in osteoblast- and osteoclast-like cells. Calcitriol treatment of a human monocyte cell line diminished the isoproterenol-stimulated cAMP production.[54] In a clonal osteogenic cell line (UMR 106-06) both PTH-, prostaglandin E_2-, calcitonin-, isoproterenol-, and forskolin-stimulated cAMP production were inhibited by treating the cells with calcitriol for 5 days.[55] It was suggested from these experiments that calcitriol modulated the AC. In ROS 17/2.8 rat osteoblast-like sarcoma cells calcitriol attenuated the PTH-stimulated AC activity. Glucocorticoids increased this activity by increasing the PTH receptor level, but the effect was abolished when the cells were treated with both steroids simultaneously, since calcitriol reduced the receptor number by approximately 80%.[56]

Another osteosarcoma cell line (SaOS-2) responded to calcitriol treatment by attenuating the PTH-, isoproterenol-, and cholera toxin-stimulated cAMP production, and it was suggested that calcitriol could alter the coupling of $G_s\alpha$ to the AC.[57] Calcitriol treatment of rat pituitary GH_4C_1 cells attenuated the vasoactive intestinal peptide (VIP)-stimulated AC activity without affecting the specific binding of ^{125}I-VIP, and effects of calcitriol were probably exerted at the level of the $G_s\alpha$ or the AC.[58]

In a breast carcinoma cell line (T47D) calcitriol increased both forskolin- and VIP-stimulated AC activity, which attenuates T47D cell growth.[59] Calcitriol treatment of the cells was associated with an increase in the amount of membrane-bound $G_s\alpha$.

In ROS 17/2.8 cells calcitriol has been shown to decrease the effect of low concentrations of PTH on PKAI activity, whereas activation of PKAII was unchanged.[60] Total PKA activation was reduced by calcitriol. It is not known if these effects were induced by upregulation of the RIIβ subunit as shown for the FRTL-5 cells.

Previous studies have shown that calcitriol may regulate the level of cAMP-dependent protein kinase inhibitors (PKIs). PKIs inhibit the phosphotransferase reaction of PKA C subunits. Calcitriol is an inhibitor of PKI activity in chicken kidneys, but had no effect in the other organs studied.[61] It is unlikely that an inhibitory effect of calcitriol on cAMP analog-stimulated FRTL-5 cell growth and iodide uptake is caused by PKI upregulation. Moreover, PKAII-stimulated iodide uptake was not attenuated in the calcitriol-treated cells.

V. SUMMARY

In the rat thyroid FRTL-5 cell line calcitriol, the biologically most active of the naturally occurring vitamin D metabolites, attenuates both TSH-stimulated cAMP production and the effects of cAMP. Calcitriol treatment abolishes the upregulation of the TSHR number occurring in cells cultivated in the absence of TSH. In addition, the level of $G_{i\text{-}2}\alpha$ increases, which may further attenuate the transmembrane signaling of TSH and facilitate the effects of IGFs. The effect of cAMP on PKAI stimulation is inhibited by increasing the level of the PKA subunit RIIβ. Regulation of TSHR, $G_{i\text{-}2}\alpha$ and RIIβ is associated with altered cell proliferation and differentiation in several cells and tissues. Effects of calcitriol on these proteins indicate how the vitamin D endocrine system may regulate cAMP signaling in both classical and nonclassical target tissues.

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